AD_____

Award Number: DAMD17-99-1-9099

TITLE: Peptide Antiestrogens for Human Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Richard J. Pietras, Ph.D., M.D.

CONTRACTING ORGANIZATION: University of California, Los Angeles
Los Angeles, California 90095-1406

REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20011212 142

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2001	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE

Peptide Antiestrogens for Human Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-99-1-9099

6. AUTHOR(S)
Richard J. Pietras, Ph.D., M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, Los Angeles
Los Angeles, California 90095-1406
E-Mail: rpietras@ucla.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Estrogen binds specific receptors that are present in 60-70% of breast cancers. The estrogen receptor (ER) is a phosphoprotein that regulates transcription and growth by binding estrogen response elements (ERE) in DNA. Inactive ER is a monomer that forms dimers on estrogen-induced phosphorylation. Transcriptional activity of ER is regulated by distinct conformational states resulting from ligand binding, and the induced signal complex recruits steroid receptor coactivator proteins, such as SRC-1, that are essential for growth. Peptides modeled from interacting sites of ER proteins may selectively inhibit ER signaling and act as antiestrogens. To test this hypothesis, we prepared small peptides to mimic a highly conserved ER sequence including tyrosine-537 and surrounding leucine residues. Peptide antiestrogens, but not control peptides, block ER association with SRC-1 and disrupt binding of ER to ERE. In *in vitro* studies, estradiol stimulates breast cell growth, and this estrogen effect is blocked by peptide antiestrogens conjugated with *Antennapedia* carrier but not by control peptides. Using *in vivo* tumor xenografts, treatment of nude mice with peptide antiestrogens shows significant activity in arresting growth of estrogen-dependent breast tumors. This work provides target validation but also shows that peptide drugs are difficult to administer. Thus, we are preparing more lipid-like, peptidomimetic derivatives that function similarly but may be easier to use in the clinic.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)

Breast Cancer, Estrogen Receptors, Antiestrogens, Peptide

15. NUMBER OF PAGES
25

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

INTRODUCTION

⁎ The estrogen receptor (ER) is a phosphoprotein found in 60-70% of human breast tumors at diagnosis (1-3). Antiestrogen therapy with tamoxifen, a partial agonist to ER, has had a significant impact on survival in patients with breast cancer, but tamoxifen also has several undesirable side-effects. The antitumor effect of antiestrogen therapy is due to close regulation of breast cell growth by estrogens. However, as breast cancer progresses, it becomes resistant to estrogens, and most patients no longer respond to current antiestrogens (1-3). The continued expression of estrogen and/or progesterone receptors in most patients with tumor progression on tamoxifen indicates that mechanisms for resistance other than receptor loss are common in breast cancer and are responsible for treatment failure (4,5).

This proposal is based on new understanding of the biology of ER, a phosphoprotein that forms a dimer required for binding to specific estrogen response elements in the nucleus, leading to promotion of breast cancer cell growth (6-8). Transcriptional activity of ER is now known to be related to the conformational state of the receptor, especially with respect to the molecular orientation of helix-12 in the ligand-binding domain of ER (8). Helix-12 contains leucine-rich regions that interact with steroid receptor coactivator proteins that, in turn, regulate transcription (9,10) (see Table 1).

TABLE 1. a) Amino acid sequence comparison of AF2 α-helix region in ER from several species, with helix-12 starting at residue 538. Conserved region is in boldface. b) Sequence comparison with ER-ß. c) Sequence alignment of nuclear receptor proteins, including progesterone and androgen receptors (9,11).

a	PL-**YDLLLEML**-DA	535-546	human ER
	PL-**YDLLLEML**-DA	539-550	mouse ER
	PL-**YDLLLEML**-DA	527-538	xenopus ER
b	PV-**YDLLLEML**-NA	433-444	human ER-ß
c	EF-PEMMSEVI-AA	904-915	progesterone receptor
	DF-PEMMAEI I-SV	889-900	androgen receptor

Manipulation of helix-12 interactions with coactivator proteins may provide alternate approaches to anti-hormone therapy. We have synthesized peptides designed to disrupt binding of estrogen receptor with coactivator proteins. Our specific aims for this project include the following experimental objectives:

1) Synthesis of small phosphotyrosyl-peptides targeted to a highly conserved sequence in estrogen receptor including tyrosine-537 and surrounding leucine residues. Experiments are planned to evaluate the efficacy of these peptide antiestrogens in antagonizing estrogen receptor activity in breast cancer cells, including block-ade of estrogen receptor dimerization, reduction of estrogen receptor association with steroid receptor coacti-vator protein and suppression of estrogen receptor binding to specific estrogen-response elements in DNA.

2) Evaluation of the antitumor efficacy *in vitro* and *in vivo* of small phosphotyrosyl- and malonyltyrosyl-peptides that suppress dimerization and DNA binding of estrogen receptor in human breast cancer cells. Alternative modes for the efficient delivery of low concentrations of peptides *in vivo* will be considered, and effects of peptide antiestrogens on bone, serum cholesterol, uterus and body composition will be evaluated in rodent models.

It is important to develop new antiestrogens which work through different mechanisms of interaction with ER, since these would likely prove useful in treatment of breast cancers that become resistant to conventional anti-estrogens. This project offers an innovative approach to antitumor therapy with the potential for developing novel antiestrogens with minimal toxicity to noncancerous tissues, and it may advance our understanding of the role of estrogen receptor in hormone action (12).

BODY: RESEARCH PROGRESS

AIM 1) Synthesis of small phosphotyrosyl-peptides targeted to tyrosine-537 and the neighboring leucine-rich region in ER and evaluation of their efficacy in the blockade of ER dimerization and ER binding to steroid receptor coactivator and DNA in human breast cancer.

1.a. Peptides disrupt binding of ER with DNA

In order to evaluate potential antiestrogen effects of peptides that mimic the initial sequence in helix-12 in ER, peptides were synthesized by established methods with N-terminal acetylation and a C-terminal amide in the UCLA/Jonsson Cancer Center Peptide Synthesis Facility (12,13). Peptide constructs were characterized by HPLC and mass spectral analysis and found to be > 95% pure. The octapeptide, pY8, contains the sequence:

  N-Pro-Leu-*pTyr-Asp-Leu-Leu-Leu-Glu-C (PLpYDLLLE)

and its nonphosphorylated analog, conY8, has the sequence :

  N-Pro-Leu-Tyr-Asp-Leu-Leu-Leu-Glu-C (PLYDLLLE).

An additional control peptide with a scrambled sequence, con8, is shown below:

  N-Val-Pro-Leu-Asp-Leu-Leu-Leu-Glu-C (VPLDLLLY).

Other peptides of varying size (5-mer and 12-mer) to ascertain the optimal preparation for use in cellular studies have also been prepared (refer to Table 2 in original proposal) (12).

Interaction of ER with nuclear ERE is prerequisite for activation of transcription. To assess specific binding of ER with ERE, we used purified recombinant human ER from MCF-7 breast cancer cells (13). A double-stranded 27-bp probe [5'-GATCCTAGAGGTCACAGTGACCTACGA-3'] encoding the Xenopus vitellogenin A_2 ERE was ^{32}P-end-labeled with polynucleotide kinase. Gel mobility shift assays for the human ER were performed as described (13). The ER in 20 mM reaction buffer (HEPES, pH 7.5, 1 mM EDTA, 100 mM Kcl, 1 mg/ml BSA, 100 nM estradiol, 15% glycerol, and proteinase inhibitors) was incubated with 500 ng of poly (dI-dC) for 15 min at 4 C, and then 20 fmol of the ^{32}P-labeled ERE probe was added for 15 min at 4 C in a total volume of 20 μl. Samples were loaded onto a pre-electrophoresed 5% polyacrylamide gel followed by electrophoresis with cooling at 175 V for 3h in 25 mM TRIS, pH 8.0 with 152 mM glycine and 1 mM EDTA. Under these conditions, the human ER reacts specifically with synthetic ERE in the gel mobility shift assay, allowing formation of an ER-ERE complex (13) (FIG. 1).
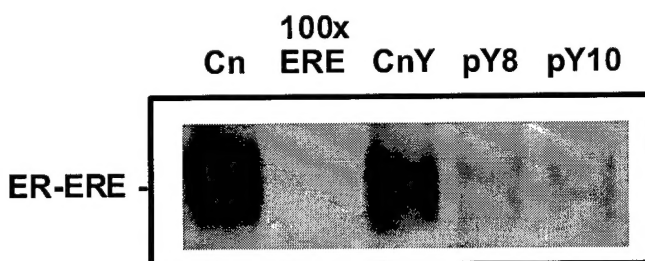

FIG. 1. Gel mobility shift assays of human ER and estrogen-responsive element (ERE). Purified human ER (60 nM) incubated with control solution (Cn), 100-fold molar excess of unlabelled ERE (100x ERE) or with peptides at 2.5 μM. ER was incubated with peptides for 15 min at 4°C, then with 100 nM estradiol-17ß and ^{32}P-ERE. Peptides included peptide pY8, control Y8 peptide (CnY), and a decapeptide, N-Val-Pro-Leu-pTyr -Asp-Leu-Leu-Leu-Glu-Met-C (pY10).

This ER-ERE interaction is blocked by competition with 2.5 μM pY8-peptide but not by competition with 5 μM conY8-peptide (43) or con8-peptide (data not shown). Thus, peptide antiestrogens disrupt ER binding to a specific ERE *in vitro*. However, the IC_{50} for this effect may exceed by 100-fold that required for other cellular actions of the peptides (see ER/SRC-1 interaction below).

1.b. Peptides interfere with ER dimerization

Experiments to assess effects of peptides on ER dimerization, using molecular sizing chromatography with Sephadex G-200 (13,14), are underway. Using this approach, we find that 25 μM pY8 inhibits dimerization of ER (12,13). As with peptide inhibition of ER binding to DNA, peptide interference with ER dimerization appears to occur at a higher IC_{50} than other cellular actions of the peptide (see ER/SRC-1 interaction below). We have confirmed these findings in the past grant year.

1.c. Peptides block molecular association between ER and steroid receptor coactivator proteins

As noted above, upon activation in vivo, ER bind to DNA response elements and recruit co-activator proteins and general transcription factors to form an active complex for stimulation of gene expression. Steroid receptor coactivator-1 (SRC-1) is a well-characterized coactivator protein (165 kd) that mediates steroid hormone responses by promoting receptor-dependent transactivation of genes (15,16), and disruption of the SRC-1 gene results in partial resistance to hormone (16). Short sequence motifs in SRC-1 and other coactivators are necessary to mediate the binding of these proteins to nuclear receptors (10). In order to assess the effect of peptide antiestrogens on the interaction between ER and SRC-1, T47D breast cancer cells were treated in vitro with or without 1nM estradiol-17ß, and cell lysates were prepared for immunoprecipitation with antibody to ER, followed by gel electrophoresis and immunoblotting with antibody to SRC-1 as before (17). In the absence of peptide antiestrogens, SRC-1 and ER form a binding complex beginning at 15 min after estrogen treatment, and the association is maximal by 30 min. Prior incubation of breast cells with pY8 interferes with this ER/SRC-1 binding (FIG. 2). In contrast, pre-treatment of T47D breast cancer cells with conY8 or con8 elicits no effect on ER/SRC-1 binding in vitro (FIG. 2) (12).
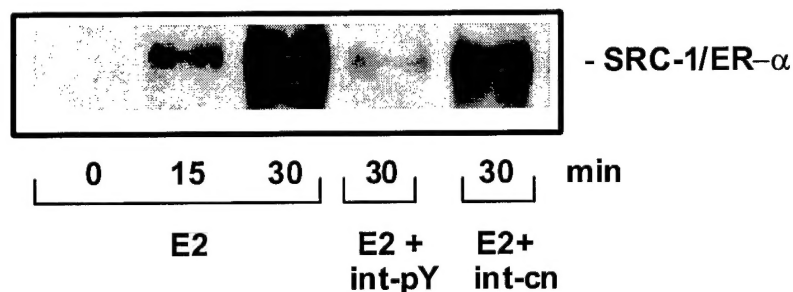

FIG. 2. Effect of estradiol-17ß (E2) on association of ER and SRC-1 in T47D cells. T47D breast cancer cells were treated in vitro with 10nM E2 or control vehicle for 15-30 min. For more efficient delivery of peptide, pY8 was coupled with a short peptide from the homeodomain of antennapedia, a vector that promotes internalization of peptides linked to its carboxy-terminus (int-pY8) (18). Int-pY8 or int-peptide control (int-cn) were added 30 min before E2 for an extra 30 min. Lysates were prepared and processed as before. Samples were immunoprecipitated with monoclonal anti-ER antibody-10 (Neomarker), followed by electrophoresis and immunoblot with monoclonal anti-SRC-1 antibody (Affinity Bioreagents).

Thus, tyrosine-537 and adjacent leucine residues in ER may be important in regulating ER/SRC-1 interaction in breast cancer. In contrast to peptide effects on ER dimerization and DNA binding in vitro, the peptide effect on ER/SRC-1 association can be elicited at nanomolar concentrations of active peptides. Thus, peptide blockade of the binding of receptor coactivator protein with ER may have more physiologic significance in vivo. These studies are now close to completion.

AIM 2) Evaluation of the antitumor efficacy in vitro and in vivo of phosphotyrosyl- and malonyltyrosyl-peptides that suppress biologic activity of ER in human breast cancers

2.a. Peptide antitumor effects *in vitro* and *in vivo*

One problem with use of phosphotyrosyl-peptides *in vitro* or *in vivo* is susceptibility of the constructs to degradation by cellular tyrosine phosphatase enzymes. To address this difficulty, we prepared phosphotyrosyl-mimic peptides that use malonic acid rather than phosphate residues at tyrosine sites. Malonyl-tyrosine residues appear to mimic the phosphotyrosine conformation in proteins and evade the action of cellular enzymes targeted to phosphotyrosine (19,20). The malonyltyrosyl-peptides contain the sequence surrounding tyrosine-537 in ER (12,13) (see Table 1). Malonyltyrosyl-octapeptide, mY8, was synthesized by established methods and contains the sequence:

N-Pro-Leu-mTyr-Asp-Leu-Leu-Leu-Glu-C   (PLmYDLLLE).

We find that malonyltyrosyl-peptide constructs, as phosphotyrosyl-peptides, suppress binding of ER to specific ERE in human breast cancer cells. This ER-ERE interaction is blocked by competition with 2.5 µM mY8-peptide but not by 5 µM conY8-peptide (12,13). Our ongoing studies indicate that 8-12-mer malonyltyrosyl-peptides are the optimal peptide sequence for use in *in vivo* studies.

Using the pY8-internalization vector (int-pY8), we find that nanomolar concentrations of the peptide have good efficacy in disruption of estrogen-induced growth of human breast cancer cells (12). The anticipated growth stimulation by estrogen is found after treatment of MCF-7 cells with control internalization peptide alone, exceeding growth of control cells in the absence of estrogen by 3-fold. Similarly, a low concentration of free pY8-peptide alone in solution (25 µM) does not alter the growth response to estrogen. However, peptide antiestrogen coupled with internalization peptide suppresses the expected growth effect of estrogen (P<0.001). A dose-response study using concentrations of drug ranging from 0.02 to 500 nM shows that the pY8-internalization peptide is effective in growth inhibition of MCF-7 cells at concentrations < 25 nM (12). Studies of breast tumor xenografts *in vivo* are also underway using methods as before (17) with peptide delivery by IP injection (13,17). However, in these experiments, we have encountered difficulty in obtaining uniform antitumor efficacy, a result that may be due to biologic variabilities in peptide administration and bioavailability.

2.b. Peptidomimetic derivatives to disrupt biologic activity of estrogen receptor

We plan to study the biologic activity of other peptide antiestrogens using identical assays for cell proliferation (12,13) (see original proposal). Depending on results of these studies, additional peptides may be designed and synthesized for testing. However, delivery of peptides in the clinic may be problemsome (24). Therefore, we are also assessing the potential use of more lipophilic derivatives that mimic the activity of peptide antiestrogens but may be easier to administer.

In collaborative studies with Dr. Michael Jung in the Department of Chemistry at UCLA, we have synthesized small steroidal and non-steroidal compounds targeted to disrupt the biologic activity of the helix 12 region in ER. The choice of our target molecules is based on structural information given in the x-ray structures of the ligand-binding domain of the estrogen receptor with both estradiol and raloxifene. One requires two OH groups (hydroxyl or phenol) placed at the right distance in order to bind to the receptor but, in addition, to cause the helix 12 to assume a different conformation, one requires also an additional binding element, namely a correctly disposed tertiary amine, to bind to Asp 351. We have prepared several steroidal and non-steroidal compounds all of which have both the required diol unit properly spaced along with a variable length alkyl chain containing a terminal tertiary amine (or guanidine unit) to bind to Asp 351 and thereby induce the conformation change in order to shift helix 12.

Candidate antiestrogens prepared in the Chemistry Laboratory are under evaluation for estrogen binding activity by use of established [^{3}H]-estradiol-17ß competition binding assays. Results of experiments showing specific binding of [^{3}H]-estradiol-17ß with human MCF-7 breast cancer cells are shown in FIG. 3. The first set of screening experiments to assess competition for [^{3}H]-estradiol-17ß binding was completed recently, with data presented in FIG. 4. Of four novel compounds tested, at least one, test compound 1, shows evidence of significant competition for specific [^{3}H]-estradiol-17ß binding with intact MCF-7 cells *in vitro*. Experiments to

measure competition of the test compounds for specific[³H]-estradiol-17ß binding with particle-free cytosol fractions from MCF-7 cells are in progress.

Compounds with significant biologic activity in these screening assays will be selected for further study of antitumor activity using MCF-7, ZR75 and T47D human breast cancer cells *in vitro*. The results of initial studies on proliferation of MCF-7 cells are shown in FIG. 5. At least one compound, test compound 1, again appears to block growth of the breast cancer cells in this *in vitro* assay, and the extent of growth inhibition exceeds that found with tamoxifen at equivalent doses.

We will further assess the most promising antiestrogens for biologic efficacy using assay methods outlined above. One primary test will be assessment of the molecular association between ER and the steroid receptor coactivator protein, SRC-1, as above. Additional screening assays will include tests for suppression of ER-induced transcriptional activity using an ERE-CAT reporter gene approach. Compounds with significant biologic activity in screening assays will be selected for further studies of antitumor activity using MCF-7 cells *in vivo*. These results will be compared with those from experiments with corresponding peptide antiestrogens.
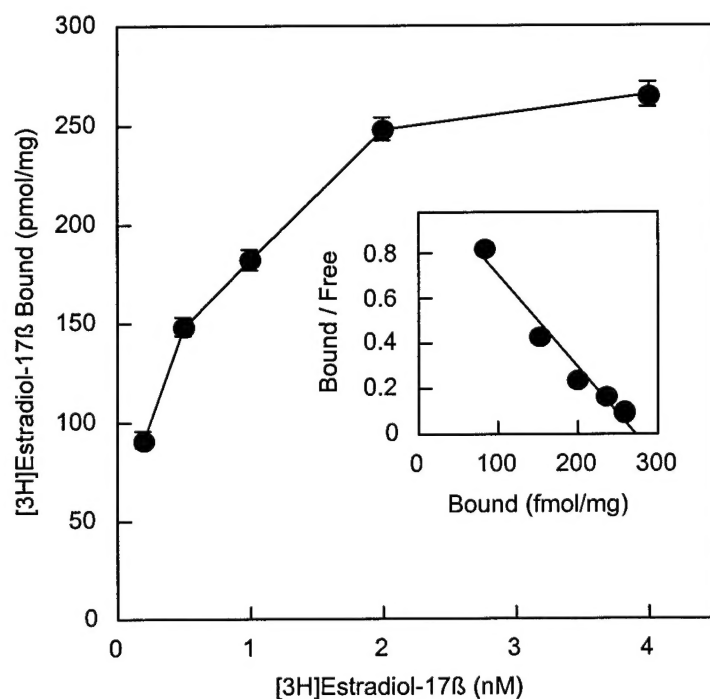

FIG. 3. Binding of [³H]estradiol-17ß by MCF-7 human breast cancer cells. Specific binding of [³H]estradiol-17ß by MCF-7 cells was determined by methods described elsewhere (14,17). A 100-fold molar excess of unlabeled estradiol-17ß was present with [³H]estradiol-17ß in paired samples for determination of displaceable binding. Specific binding of estradiol by intact cells is shown. Scatchard analyses of the binding data to determine estrogen-binding capacity (B_{max}) and the affinity of hormone binding (K_d) are shown in the inset. The K_d of estradiol binding to MCF-7 cells was 2.5×10^{-10} M, and the estradiol binding capacity in MCF-7 cells was 270 fmol/mg protein. These values are based on results from three experiments.
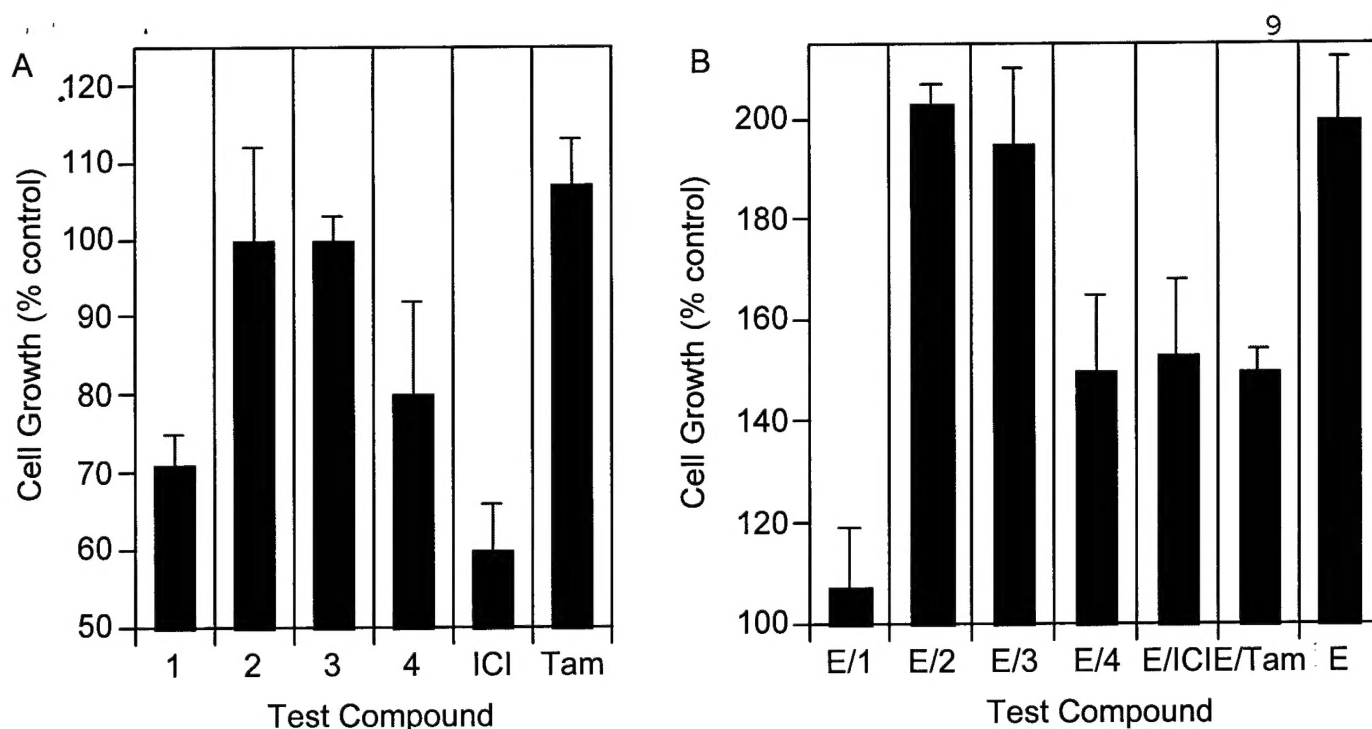
FIG. 5. Effects of test compounds 1-4, estradiol-17ß, ICI 182,780 and tamoxifen on growth of MCF-7 cells *in vitro*. Growth assays were done as before (17). Test compounds were administered at 1 μM, without (Panel A) or with (panel B) estradiol-17ß (E) at 10 nM. As additional controls, we also used ICI 182,780 (ICI) at 10 nM and tamoxifen (Tam) at 1μM. The results are from one set of duplicate experimental determinations. Further experiments will be required.

2.c. Tissue selectivity of synthetic antiestrogens

Estrogen is a key regulatory hormone which, in addition to its role in reproductive tissues, affects a number of physiological systems, including the skeleton and cardiovascular system. In order to evaluate tissue selectivity of the final peptide and peptidomimetic steroidal formulations, these agents will be given to immature female mice for 3 days, and effects on uterine wet and dry weight will be assessed (23). In addition, uterine hypertrophy will be evaluated in older (up to 15-mo-old) female mice treated with peptides for 28-90 days. Effects of peptides on total serum cholesterol, fat body mass and lean body mass in these aged female rodents will be tested. In ovariectomized female mice, we plan to assess effects of peptides on ovariectomy-induced increments in body weight gain, total serum cholesterol, and bone loss. Following ovariectomy, rodents will be assigned to control groups including both placebo and positive control with estrogen replacement using established methods (23). Our goal is to develop novel antitumor agents with minimal toxicity to noncancerous tissues, and these experiments will help to establish the response profile and tissue selectivity of peptide antiestrogens.

KEY RESEARCH ACCOMPLISHMENTS

- Small leucine-rich peptides that mimic ER sequence at the start of helix-12 reduce the formation of ER homodimers and reduce binding of ER to ERE.

- Small leucine-rich peptides that mimic ER sequence at the start of helix-12 suppress association of ER with SRC-1.

- Estrogen-dependent growth of human breast cancer cells is blocked by pre-treatment with small leucine-rich peptides that mimic ER sequence at the start of helix-12.

REPORTABLE OUTCOMES

Presentations

1. "Small Molecule Inhibitors of Estrogen Receptor Function". Presented at Molecular Oncology Seminar, Genentech, South San Francisco (1999).

2. "Peptide antagonists of the estrogen receptor block growth of human breast cancer cells". Presented at Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta (2000).

3. "Interactions between Type I receptor tyrosine kinases and steroid hormone receptors : Therapeutic implications". Presented at First International Symposium on Translational Research in Oncology, Dublin, Ireland (2001).

Abstracts

1. Pietras, R.J., Marquez, D., Chen, X. and Li, D. (2000). Peptide antagonists of the estrogen receptor block growth of human breast cancer cells. Era of Hope DOD Breast Cancer Research Program Proceedings, **2**: 535.

Publications

1. Pietras, R.J., Nemere, I. and Szego, C.M. (2001). Steroid hormone receptors in target cell membranes. Endocrine **14** : 417-427.

No patents, degrees, development of cell lines, informatics or additional funding or research opportunities to be reported at this time.

CONCLUSIONS

This project is a new approach to antitumor therapy with the potential for developing antiestrogen treatments with minimal toxicity to noncancerous tissues. Small leucine-rich peptides that mimic ER sequence at the start of helix-12 in the receptor molecule are especially effective in suppressing the association of ER with SRC-1. This molecular action appears to elicit blockade of breast cancer cell proliferation. The results of our preliminary studies suggest that treatment with small peptide antiestrogens may prove more effective than drugs currently available in blocking the growth-promoting signals of estrogen receptors. Further studies to assess the optimal constructs and the safety of these peptide compounds are planned. This work provides good evidence of target validation for helix-12 in estrogen receptor and may allow preparation of other derivatives with biologic activity at this site.

REFERENCES

1). Harris J, M Lippman, U Veronesi and W Willett (1992). Breast cancer. New Engl. J. Med., **327** : 473-480.

2.) Katzenellenbogen BS, M Montano, K Ekena, M Herman and E McInerney (1997). Antiestrogens: Mechanisms of action and resistance in breast cancer. Breast Cancer Res. Trtmt.,**44** : 23-38.

3.) Tonetti D and VC Jordan (1997). The role of estrogen receptor mutations in tamoxifen-stimulated breast cancer. J. Steroid Biochem. Mol. Biol., **62**: 119-128.

4.) Encarnacion C, D Ciocca, W McGuire, G Clark, S Fuqua and C Osborne (1993). Measurement of steroid hormone receptors in breast cancer patients on tamoxifen. Breast Cancer Res. Trtmt., **26**: 237-246.

5.) Mullick A and P Chambon (1990). Characterization of the estrogen receptor in two antiestrogen-resistant cell lines, LY2 and T47D. Cancer Res. **50**: 333-338.

6.) Magliaccio A, A Rotondi and F Auricchio (1986). Estradiol receptor: phosphorylation on tyrosine in uterus and interaction with anti-phosphotyrosine antibody. EMBO J., **5** : 2867-2872.

7.) Brandt M. and L. Vickery (1997). Cooperativity and dimerization of recombinant human estrogen receptor hormone-binding domain. J. Biol. Chem., 272: 4843-4849.

8.) Brzozowski A., A. Pike, Z. Dauter, R. Hubbard, T. Bonn, O. Engstrom, L. Ohman, G. Greene, J. Gustafsson and M. Carlquist (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature, **389**: 753-758.

9.) Danielian P, R White, L Lees and M Parker (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J., **11**: 1025-1033.

10.) Heery D., E. Kalkhoven, S. Hoare and M. Parker (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature, 387: 733-736.

11.) White R, M Sjoberg, E Kalkhoven and MG Parker (1997). Ligand-independent activation of the oestrogen receptor by mutation of a conserved tyrosine. EMBO J., **16** : 1427-1435.

12.) Pietras RJ, D Marquez, X Chen and D Li (2000). Peptide antagonists of the estrogen receptor block growth of human breast cancer cells. Era of Hope DOD Breast Cancer Research Program Proceedings, **2** : 535.

13.) Pietras RJ and J Lee (1997). Antitumor effect of phosphotyrosyl-peptides that block binding of estrogen receptor to DNA in human breast cancer cells. Proc.Am. Assoc. Cancer Res., **38**: 174.

14.) Pietras RJ and CM Szego (1980). Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membrane. Biochem. J., **191** : 743-760.

15.) Onate S., V. Boonyaratanakornkit, T. Spencer, S. Tsai, M.-J. Tsai, D. Edwards and B. O'Malley (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. J. Biol. Chem., **273**: 12101-12108.

16.) Xu J., Y. Qiu, F. DeMayo, S. Tsai, M.-J. Tsai and B. O'Malley (1998). Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science, **279**: 1922-1925.

17.) Pietras RJ, J Arboleda, D Reese, N Wongvipat, M Pegram, L Ramos, C Gorman, M Parker, M Sliwkowski, and D Slamon (1995). HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene, **10** : 2435-2446.

18.) Bonfanti M, S Taverna, M Salmona, M D'Incalci and M Broggini (1997). p21WAF1-derived peptides linked to an internalization peptide inhibit human cancer cell growth. Cancer Research, **57** : 1442-1446.

19.) Kole H, M Akamatsu, B Ye, X Yan, D Barford, P Roller and T Burke, Jr. (1995). Protein tyrosine phosphatase inhibition by a peptide containing the phosphotyrosyl mimetic L-O-malonyltyrosine. Biochem. Biophys. Res. Commun., **209**: 817-822.

20.) Ye B, M Akamatsu, S Shoelson, G Wolf, S Giorgetti-Peraldi, X Yan, P Roller and T Burke, Jr. (1995). L-O-(2-Malonyl)tyrosine: a new phosphotyrosyl mimetic for the preparation of src homology 2 domain inhibitory peptides. J Med Chem, **38** : 4270-4275.

21.) Coradini D, A Biffi, V Cappelletti and G Di Fronzo (1995). Influence of different combinations of tamoxifen and toremifene on estrogen receptor-positive breast cancer cell lines. Cancer Detection Prevention, **19**: 348-354.

22.) Couillard S, M Gutman, C Labrie, A Belanger, B Candas and F Labrie (1998). Comparison of the effects of the antiestrogens EM-800 and tamoxifen on the growth of human breast ZR-75-1 cancer xenografts in nude mice. Cancer Res.,**58**: 60-64.

23.) Ke H, V Paralkar, W Grasser, D Crawford, H Qi, H Simmons, C Pirie, K Chidsey-Frink, T Owen, S Smock et al. (1998). Effects of CP-336,156, a new nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. Endocrinology, **139**: 2068-2076.

24.) McDonnell D, Chang, C-Y and JD Norris (2000). Development of peptide antagonists that target estrogen receptor-cofactor interactions. J. Steroid Biochem. Molecular Biol. **74** : 327-335.

© 2001 by Humana Press Inc.

· Steroid Hormone Receptors in Target Cell Membranes

Richard J. Pietras,[1] Ilka Nemere,[2] and Clara M. Szego[3]

[1]Department of Medicine/Hematology-Oncology and Jonsson Comprehensive Cancer Center, University of California School of Medicine, Los Angeles, CA; [2]Department of Nutrition & Food Science and the Biotechnology Center, Utah State University, Logan, UT; and [3]Department of Molecular, Cell & Developmental Biology, the Molecular Biology Institute and Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA

Numerous reports of rapid steroid hormone effects in diverse cell types cannot be explained by the generally prevailing theory that centers on the activity of hormone receptors located exclusively in the nucleus. Cell membrane forms of steroid hormone receptors coupled to intracellular signaling pathways may also play an important role in hormone action. Membrane-initiated signals appear to be the primary response of the target cell to steroid hormones and may be prerequisite to subsequent genomic activation. Recent dramatic advances in this area have intensified efforts to delineate the nature and biologic roles of all receptor molecules that function in steroid hormone–signaling pathways. This work has profound implications for our understanding of the physiology and pathophysiology of hormone actions in responsive cells and may lead to development of novel approaches for the treatment of many cell proliferative, metabolic, inflammatory, reproductive, cardiovascular, and neurologic defects.

Key Words: Steroid hormone action; plasma membrane; receptor.

Introduction

The broad physiologic effects of steroid hormones in the regulation of growth, development, and homeostasis have been known for decades. Often, these hormone actions culminate in altered gene expression *(1)*, which is preceded by nutrient uptake and other preparatory changes in the synthetic machinery of the cell *(2)*. Owing to certain homologies of molecular structure, specific receptors for steroid hormones, vitamin D, retinoids, and thyroid hormone are often considered a receptor superfamily. The actions of ligands in this steroid receptor superfamily are commonly postulated to be mediated by receptors in the cell nucleus. On binding ligand, nuclear receptors associate with target

genes and permit selective transcription. This genomic mechanism is generally slow, often requiring hours or days before the consequences of hormone exposure are evident. However, steroids also elicit rapid cell responses, often within seconds. The time course of these acute events parallels that evoked by peptide agonists, lending support to the conclusion that they do not require precedent gene activation *(2–5)*. Rather, many rapid effects of steroids, which have been termed *nongenomic*, appear to be owing to specific recognition of hormone at the cell membrane. Although the molecular identity of binding sites remains elusive and the signal transduction pathways require fuller delineation, there is mounting evidence that steroid action is initiated by plasma membrane receptors.

A current challenge is to determine the relation of rapid responses to steroid hormones to intermediate and long-term effects. Some questions that arise in this context include the following: Is specific membrane binding responsible merely for cellular entry of the hormone? Do plasmalemmal receptors escort ligand to the nucleus? Are the membrane binding sites coupled to rapid signal transduction systems that also act in concert with nuclear transcription factors? Are the membrane receptors identical to nuclear receptors, modified forms, or entirely different entities? This review explores these important issues. In preparing this work, more than 1200 references providing significant evidence for rapid steroid actions and for membrane forms of steroid receptors were identified. Only a fraction of these citations can be presented here, and the reader is referred to several recent reviews in this area *(3–7)*.

Estrogens

As with other steroid hormones, biologic activities of estrogen in breast, uterus, and other tissues are considered to be fully mediated by a specific high-affinity receptor in cell nuclei. Estrogens are accumulated and retained in responsive cells, and it has been commonly assumed that the steroid diffuses passively to intracellular receptors. However, estradiol is a lipophilic molecule that partitions deep within the hydrocarbon core of lipid bilayer membranes, even those devoid of relevant receptors *(3)*. Several investigations now demonstrate that steroid hormones enter target cells by a membrane-mediated process that is saturable

Table 1
Brief Chronology of Selected Reports Documenting Occurrence and Activity of Membrane Steroid Hormone Receptors[a]

Steroid	Year	Observation	Reference
Estradiol	1967	Elevation of uterine cAMP by estrogen within seconds	11
	1975	Rapid endometrial cell calcium mobilization by estrogen	9
Corticosterone		Binding to plasma membranes of rat liver	108
Estradiol	1976	Effects on electrical activity of neurons	20
	1977	Specific plasma membrane binding sites for estrogen	16
Cortisol		Electrophysiologic effects on neurons	21
Progesterone	1978	Induction of oocyte maturation by steroid linked to a polymer	29
Estradiol	1979	Increased proliferation of cells with membrane ER	17
	1980	Molecular properties of ERs in liver plasma membrane	13
Vitamin D	1981	Rapid intestinal cell calcium uptake	109
Progestin	1982	Specific binding to oocyte surface and role in meiotic maturation	30
		Steroid receptor of 110 kDa on oocyte surface by photoaffinity labeling	31
Corticosterone	1983	Binding to synaptic plasma membranes	50
Estradiol	1983	Increase in density of microvilli at endometrial cell surface within seconds	112
	1984	Primary internalization of ER in endometrial plasma membrane vesicles	104
Thyroid hormone	1985	Characterization of plasma membrane binding sites	47
Estradiol	1986	High-affinity binding sites in breast cancer cell plasma membranes	26
		Altered breast cell membrane potential, density of microvilli within seconds	110
Glucocorticoid	1987	Correlation between membrane receptor and apoptosis in lymphoma cells	53
Vitamin D	1989	Rapid activation of phospholipase C (PLC) in rat intestine	5,14
		Activation of calcium channels in osteoblasts	63
Thyroid hormone		Rapid induction of glucose uptake	42
Progesterone	1990	Stimulation of calcium influx in human sperm	33
	1991	Calcium uptake mediated by sperm cell surface-binding sites	
		Action at plasma membrane of human sperm	34
Corticosterone		Correlation of neuron membrane receptors with behavior in newts	51
Aldosterone		Rapid effects on Na^+/H^+ exchange	111
Glucocorticoid	1993	Antigenic similarity between membrane and intracellular receptors	54
Estradiol		Binding and stimulation of HER-2 membrane receptor	90
	1994	Activation of adenylate cyclase signaling pathways	12
Vitamin D		Isolation of a plasma membrane receptor from chick intestine	88
Aldosterone		Identification of membrane receptor in human lymphocytes	86
Estradiol	1995	Membrane receptor with antigenic identity to nuclear receptor	7,78
		Greater nongenomic responses of membrane receptor–enriched neural cells	
Androgen		Rapid increase in cytosolic Ca^{++} in Sertoli cells	36
Estradiol	1997	Membrane action and PLC regulation	14
		Isolation of membrane binding-proteins from rat brain	81
Vitamin D	1998	Blocking of hormone activation of PKC by antibody to membrane receptor	65
Estradiol	1999	Rapid Ca^{++} mobilization required for activation of MAPK	10
		Rapid actions in neurons from ERα knockout mice	94
		Reduction of membrane ER expression by antisense to nuclear ER	80
		Membrane and nuclear ERα, and ERβ, each expressed from single transcript	25
		Activation of G-proteins, IP_3, adenylate cyclase, and MAPK by membrane ER	
Androgen		Rapid activation of MAPK pathway in prostate	37
Progesterone		Cloning and expression of binding protein from liver microsomal membrane	85
Vitamin D	2000	Ligand-induced nuclear translocation of plasma membrane receptor	89
Estradiol		Surface receptor in endothelial cells recognized by monoclonal ERα antibody	79
		Interaction of ERα with regulatory subunit of phosphatidylinositol-3-OH kinase	113
		Rapid tyrosine phosphorylation of Raf-1 and activation of MAPK resulting in prolactin gene expression in pituitary cells	114

[a]More than 1200 publications on membrane steroid receptors have appeared in the past 30 yr. Of these, only representative examples are listed here. The potential roles of alternate (25) or variant (56) forms of steroid hormone receptors and other membrane-signaling molecules (90,94) remain to be clarified.

and temperature dependent (3,8). Moreover, it is well established that estrogen can trigger in target cells rapid surges in levels of intracellular messengers, including calcium (9, 10) and cyclic adenosine monophosphate (cAMP) (11,12), as well as activation of mitogen-activated protein kinase (MAPK) (13) and phospholipase (14) (Table 1). These data have led to a growing consensus that the traditional genomic model of estrogen action does not explain the rapid effects of estrogens and must be expanded to include membrane receptors as a component of cell signaling (2–7,15).

The first unequivocal evidence for specific membrane-binding sites for estradiol-17β (E$_2$) was reported in 1977 *(16)*. Intact uterine endometrial cells equipped with estrogen receptor (ER), but not ER-deficient control cells, bound to an inert support with covalently linked E$_2$. In addition, target cells that bound could be eluted selectively with free hormone, and cells so selected exhibited a greater proliferative response to estrogens than cells that did not bind *(17,18)*. Further investigations have continued to provide compelling evidence for the occurrence of a plasma membrane form of ER and support for its role in mediating hormone actions *(3)* (Table 1).

Selye *(19)* first demonstrated that steroids at pharmacologic concentrations elicit acute sedative and anesthetic actions in the brain. However, electrical responses to *physiologic* levels of E$_2$ with rapid onset have since been reported in nerve cells from different brain regions *(4,20,21)*. Similarly, certain vasoprotective effects of estrogen appear attributable to membrane receptors *(15,22)*. Estrogen-induced release of uterine histamine *in situ* has long been associated with rapid enhancement of the microcirculation by a process that excludes gene activation *(2)*. Reinforcing these observations are new data detailing the role of nitric oxide (NO) in vascular regulation by estrogen. Normal endothelium secretes nitric oxide, which relaxes vascular smooth muscle and inhibits platelet aggregation. Estrogens elicit abrupt liberation of NO by acute activation of endothelial NO synthase without altering gene expression, a response that is fully inhibited by concomitant treatment with specific ER antagonists *(23)*. This estrogenic effect may be mediated by a receptor localized in caveolae of endothelial cell membranes *(24)*. Such observations require extension, because several independent cell-signaling complexes that appear to participate in signal transduction to the nucleus also associate with caveolar structures *(2,3,22)*.

Estrogen deficiency is associated with significant bone loss, and research on the potential role of membrane ERs in regulating bone mass has increased. Evidence for membrane-binding sites and acute effects of estrogen with an onset within 5 s has been reported in both osteoblasts and osteoclasts *(5,13)*. The effects of estrogens on bone homeostasis also appear to involve rapid activation of MAPK *(13)*, as has also been demonstrated in certain other target cells *(10,15,25)*.

When exposed to E$_2$ conjugated to fluorescein-labeled bovine serum albumin (BSA), human breast cancer cells exhibit specific surface staining *(7,26)*. Since E$_2$-BSA is considered membrane impermeant, these conjugates, developed primarily for use as immunogens and for affinity purification of nuclear ERs, have also been used to assess the membrane effects of estrogen. However, in light of the fact that E$_2$-BSA is unstable in solution, especially in the presence of cells and their enzymic products, and releases measurable amounts of free steroid *(27)*, data relying only on the use of estradiol conjugates to test for membrane effects of steroids need especially careful scrutiny. It is clear that more stable, cell-impermeant derivatives of estradiol should be developed for evaluating membrane receptors.

Progestogens and Androgens

As documented for estrogens, several physiologic effects of progestogens and androgens appear to be regulated, in part, by membrane receptors. Progesterone controls components of reproductive function and behavior. Some of these activities are mediated by interaction with neurons in specific brain regions, and membrane effects appear to be important in this process *(4,28)*. Meiosis in amphibian oocytes is initiated by gonadotropins, which stimulate follicle cells to secrete progesterone. The progesterone-induced G$_2$/M transition in oocytes was among the first convincing examples of a steroid effect at plasma membrane, since it could be shown that exogenous, but not intracellularly injected, progesterone elicited meiosis and that many progesterone-stimulated changes occurred even in enucleated oocytes *(29–32)*. Moreover, this process may be related to progesterone-induced increments in intracellular Ca^{++} and release of diacylglycerol species that elicit a cascade of further lipid messengers *(32)*.

Progesterone elicits rapid effects on membrane receptors, second messengers, and the acrosome reaction in human sperm *(33–35)*. Assay of acute sperm responses to progesterone in subfertile patients is highly predictive of fertilizing capacity *(35)*. Effects of the steroid, present in the cumulus matrix surrounding the oocyte, appear to be mediated by elevated intracellular Ca^{++}, tyrosine phosphorylation, chloride efflux, and stimulation of phospholipases, effects attributed to activation of a membrane-initiated pathway. Indeed, two different receptors for progesterone, apparently distinct from genomic ones, have been identified at the surface of human spermatozoa *(35)*; nevertheless, a monoclonal antibody (MAb) against the steroid-binding domain of human *intra*cellular progesterone receptor (PR) inhibits progesterone-induced calcium influx and the acrosome reaction in sperm *(35)*.

As with estrogens and progestogens, androgens promote a rapid increase in cytosolic Ca^{++} in their cellular targets *(36)*. Other effects of androgens that are not attributable to genomic activation include acute stimulation of MAPK in prostate cancer cells *(37)*. The androgen, 5β-dihydrotestosterone, induces vasodilation of aorta, which may be owing to direct action of the steroid on membranes of smooth muscle cells leading to modulation of calcium channels *(38)*. In osteoblasts, membrane receptors for androgen appear to be coupled to phospholipase C (PLC) via a pertussis toxin–sensitive G-protein that, after binding testosterone, mediates rapid increments in intracellular calcium and inositol triphosphate (IP$_3$) *(39)*. Of note, Benten et al. *(40)* report that testosterone elicits Ca^{++} mobilization in macrophages that lack intracellular androgen receptor (AR). These cells

express an apparent G-protein-coupled AR at the cell surface that undergoes agonist-induced internalization.

Thyroid Hormones

Thyroid hormones are well known to regulate energy expenditure and development, and membrane-initiated effects may contribute to these responses. Triiodothyronine (T_3) rapidly stimulates oxygen consumption and gluconeogenesis in liver (41). T_3 also promotes an abrupt increase in uptake of the glucose analog, 2-deoxyglucose, in responsive tissues by augmenting activity of the plasma membrane transport system for glucose (42). In rat heart, T_3 elicits a positive inotropic effect, increasing left ventricular peak systolic pressure, as early as 15 s after hormone (43). In each tissue investigated, alterations in intracellular Ca^{++} induced by thyroid hormone appear to modulate signal transduction to the cell interior (41–44).

Membrane-initiated effects of T_3 have been documented in bone cells by means of inositol phosphate signaling (45), and in brain through calcium channel activation (46). T_3 can also influence other cell processes, including the exocytosis of hormones and neurotransmitters (46), rapid effects that may be attributable to mediation by membrane receptors (44). Although uptake of T_3 can occur concomitantly with receptor-mediated endocytosis of low-density lipoprotein, and likely accompanied by carrier proteins, uptake of T_3 itself has also been reported to occur in numerous tissues by means of a high-affinity, stereospecific, and saturable process (45,47,48), as found for steroid hormones (3,8).

Glucocorticoids

In addition to their long-established effects on mobilization of energy sources by promoting catabolism and the induction of enzymes involved in gluconeogenesis, glucocorticoids have profound effects on neuron signaling and on induction of apoptosis in lymphocytes, phenomena that appear to be membrane-initiated events. Kelly et al. (21) found that glucocorticoids rapidly altered neuron-firing patterns, and many studies have verified these effects (4,6,28). These molecular events lead to glucocorticoid modulation of specific brain functions, such as the rapid response of hypothalamic somatostatin neurons to stress (49). Such abrupt changes in neuron polarization are reinforced by findings of specific, saturable binding of corticosterone to neuron membranes (50,51). Specific, high-affinity corticosterone binding to calf adrenal cortex plasma membrane is also identified by use of the biologically active radioligand [^{3}H]corticosterone (52).

Glucocorticoids also play an important role in the regulation of immune function and inflammation. In lymphoproliferative diseases, glucocorticoids are in wide use as therapeutic agents, but the cellular mechanism leading to the therapeutic effect remains unclear. In several studies using both cell lines and freshly prepared leukemia or lymphoma cells, the presence of a membrane receptor for glucocorticoids has been implicated in modulating apoptosis and cell lysis (7,53–55). Moreover, in lymphocytes, the membrane-binding site is antigenically related to the intracellular glucocorticoid receptor (iGR) and may be a natural splice variant form of the intracellular receptor (7,55,56). A potential parallel to the ER transfected in Chinese hamster ovary (CHO) cells (25) is evident.

Aldosterone and Digitalis-Like Steroids

Beyond its classic functions of promoting renal reabsorption of sodium and excretion of excess potassium, aldosterone enhances sodium absorption from colon and urinary bladder. In each tissue, the mineralocorticoid effect is owing to enhanced activity of amiloride-sensitive sodium channels. Aldosterone rapidly augments Na^+/H^+ exchange (6,57). This function is Ca^{++}- and protein kinase C (PKC)-dependent but independent of nuclear receptor activation, transcription, and protein synthesis (6,58). Similarly, "non-genomic" action of aldosterone has also been reported to underlie its acute effects on cardiac function and on sodium transport in vascular smooth muscle cells (6,58).

Digitalis-like compounds are often forgotten members of the steroid superfamily. These plant-derived agents elicit inotropic and chronotropic effects on the heart but also affect many other tissues. Endogenous steroidal ligands, termed *digitalis-like* or *ouabain-like* factors, have been found in sera of humans and other animals with blood volume expansion and hypertension (59,60) and may be released from adrenal cortex (60). These ligands elicit inhibition of membrane-associated Na^+,K^+-ATPase, likely the principal receptor for these agonists. It is notable that the steroid-binding domain of Na^+,K^+-ATPase and that of nuclear hormone receptors share significant amino acid sequence homology (61). In addition to membrane actions of these compounds on Na^+,K^+-ATPase, ouabain-induced hypertrophy in myocytes is accompanied by promotion of Ca^{++} flux and initiation of protein kinase–dependent pathways leading, in turn, to specific changes in transcription and altered expression of early response- and late-response genes (62). Thus, the biologic effects of digitalis-like compounds, long considered the exception to the concept of exclusive genomic influence, may render them more closely integrated with the steroid hormone superfamily than was previously recognized.

Vitamin D Metabolites

Membrane-initiated effects of the seco-steroid hormone, 1,25-dihydroxyvitamin D_3 (1,25[OH]$_2$D$_3$), are well documented in bone and cartilage. In osteoblasts, Caffrey and Farach-Carson (63) elucidated possible connections between rapid effects of 1,25(OH)$_2$D$_3$, requiring milliseconds to minutes, and longer-term effects owing to gene expression. Their laboratory was the first to show activa-

tion of calcium channels by $1,25(OH)_2D_3$ *(63)*. Calcium, which can signal gene expression through multiple pathways, promotes key phosphorylation events in certain bone proteins *(5)*. Osteoblasts exhibit rapid changes in IP_3 and diacylglycerol in response to vitamin D metabolites via activation of PLC *(5,14)*. Other bone cells with rapid responses to vitamin D metabolites include osteosarcoma cells and chondrocytes *(5,64)*. The latter system is particularly intriguing because chondrocytes elaborate matrix vesicles that appear critical in bone mineralization. The matrix vesicles, which lack nuclei, exhibit specific, saturable binding of $1,25(OH)_2D_3$, especially when derived from growth zone chondrocytes *(65)*.

Other rapid effects of vitamin D occur in a variety of cell types. Muscle cells respond within seconds to $1,25(OH)_2D_3$ via several mediators that alter cardiac output in some instances, while acute activation of calcium channels in skeletal muscle promotes contraction *(5,66)*. Of note, in lymphoproliferative disease, $1,25(OH)_2D_3$ appears to prime monocytic leukemia cells for differentiation through acute activation or redistribution of PKC, Ca^{++}, and MAPK *(5, 67)*. In pancreas and intestine, activation of membrane-associated signaling pathways results in vesicular exocytosis. Pancreatic β-cells respond to $1,25(OH)_2D_3$ with enhanced intracellular Ca^{++} coupled to increased insulin release *(68)*. In intestine, $1,25(OH)_2D_3$ stimulates exocytosis of transported vesicular calcium and phosphate. These cellular events may be related to vitamin D–promoted alterations in the levels of α-tubulin *(5)*, thereby influencing assembly of microtubules and possibly providing a means for vectorial transport of absorbed ions. Several signal transduction pathways have been found to respond rapidly to exogenous $1,25(OH)_2D_3$, including activation of protein kinases and promotion of abrupt increments in Ca^{++}, but integration of these signaling cascades with the physiologic response of enhanced ion absorption remains to be established *(5,68,69)*.

Investigations with vitamin D congeners have recently indicated the potential hormonal nature of $24,25(OH)_2D_3$, once thought to represent merely the inactivation product of precursor $25(OH)D_3$. Acute effects of $24,25(OH)_2D_3$ have been observed in bone cells and in intestine; $24,25(OH)_2D_3$ also inhibits rapid actions of $1,25(OH)_2D_3$ *(5)*. This may explain why abrupt effects of $1,25(OH)_2D_3$ often fail to be observed in vivo *(70)*: normal, vitamin D–replete subjects have endogenous levels of $24,25(OH)_2D_3$ sufficient to inhibit acute stimulation of calcium transport by $1,25(OH)_2D_3$, thus providing a feedback regulation system *(69)*.

Retinoids

Retinoic acid exerts diverse effects in the control of cell growth during embryonic development and in oncogenesis. It is widely considered that effects of retinoids are mediated through nuclear receptors, including those for retinoic acid as well as retinoid X receptors *(1)*. However,

other retinoid response pathways appear to exist, independent of nuclear receptors *(71)*. Cellular uptake of retinol (vitamin A) may involve interaction of serum retinol-binding protein with specific surface membrane receptors followed by ligand transfer to cytoplasmic retinol-binding protein *(72)*. In this regard, targeted disruption of the gene for the major endocytotic receptor of renal proximal tubules, megalin, appears to block transepithelial transport of retinol *(73)*. It is noteworthy that megalin may also be implicated in receptor-mediated endocytosis of $25(OH)D_3$ in complex with its plasma carrier *(74)*. In addition, retinoic acid binds mannose-6-phosphate (M6P)/insulin-like growth factor-2 (IGF-2) receptor with moderate affinity and appears to enhance its receptor activity *(75)*. M6P/IGF-2 receptor is a membrane glycoprotein that functions in binding and trafficking of lysosomal enzymes, in activation of transforming growth factor-β, and in degradation of IGF-2, leading to suppression of cell proliferation. The concept of multiple ligands binding to and regulating the function of a single receptor is relatively novel but has important implications for modulating and integrating the activity of seemingly independent biologic pathways.

Properties of Membrane Receptors for the Steroid Superfamily

Despite renewed interest in membrane steroid receptors, the physical identity of receptors with high binding affinity for ligand remains elusive. Isolation and structural characterization of these molecules remains to be accomplished. They may be known membrane components (e.g., enzymes, ion channel subunits, receptors for nonsteroid ligands), with previously unrecognized binding sites for steroids, new forms of steroid hormone receptors, "classic" receptors complexed with other membrane-associated proteins, or truly novel membrane proteins.

Estrogens and Progestogens

Efforts to isolate and purify membrane receptors that mediate rapid effects of steroids are under way in several laboratories (Table 2). Early work on purification of ER from uterus and liver plasma membranes suggested that it was a protein species with high-affinity, saturable binding specific for estradiol-17β *(16,18)*. The molecular size of solubilized receptor was in the range of intracellular ER *(18,76)*. Other work to isolate plasma membrane estrogen-binding proteins identified the 67-kDa species characteristic of nuclear receptor, but additional proteins of variant size ranging from 28 to 200 kDa were also revealed *(77)*. To determine whether membrane ER had antigenic homology with nuclear ER, Pappas et al. *(78)* used antibodies prepared to different functional epitopes of intracellular receptor and demonstrated surface labeling in nonpermeabilized rat pituitary cells by confocal scanning laser microscopy. Recent work by Russell et al. *(79)* has demonstrated, by means

Table 2

Representative Examples of Physical Properties of Membrane-Associated Receptors for Ligands of Steroid Hormone Superfamily[a]

Ligand	MW (kDa)	K_d (M)	Binding capacity (fmol/mg protein)	Homology with nR	Tissue	Reference
Estradiol	$51–78^b$ $105–148^c$	2.8×10^{-10}	526	ND	Rat hepatocytes	18
	11–67	3.6×10^{-10}	370	ND	Rabbit uterus	77
	67			Yes	CHO cell (ER transfected)	25
Progestin	110	5×10^{-7}		ND	Amphibian oocyte	30
	110	1×10^{-6}		ND		31
	28,56	6.9×10^{-8}	Variable	ND	Porcine liver	84
Vitamin D	65	7×10^{-10}	240	No	Chick intestine	88
		1.7×10^{-11}	124	No	Rat growth chondrocytes	65
		2.8×10^{-11}	100	No	Rat resting chondrocytes	
	36	1×10^{-8}		ND	Rat osteoblast-like cells	87
Aldosterone	50	1.1×10^{-8}	350	No	Pig liver	86
Glucocorticoids		1×10^{-7}		ND	Rat synapses	50
	97–150	2.4×10^{-7}	384	Yes	S-49 lymphoma cells	55
		5.1×10^{-10}		ND	Amphibian synapses	51
Thyroid hormone	145	2×10^{-9}	320	No	Human placenta	47
		6×10^{-10}		ND	Rat myoblasts	48

[a]Only representative examples of steroid-binding membrane macromolecules are presented here. Please refer to text for additional references. Homology of membrane macromolecules to nuclear receptor forms (nR) is noted; MW, apparent molecular weight; ND, not determined.

[b]High salt (0.4 M KCl).

[c]Low salt (0.01 M KCl).

of monoclonal anti-ERα, that human endothelial cells possess surface-binding sites for estrogen (see Table 1). In evaluating the source and distribution of membrane ER, target cells with expression of ERα were treated with antisense oligonucleotide to nuclear ERα to suppress expression of receptor protein (80). This approach significantly reduced expression of membrane as well as nuclear forms of ER. Using an alternate method to assess receptor origin, Razandi et al. (25) transfected cDNA for ERα and ERβ into CHO cells, which do not normally express ER. The transfections resulted in ER expression in both nuclear and membrane fractions, suggesting that membrane and nuclear ER are derived from a single transcript. In addition, both ERα and ERβ were expressed in membranes, and both receptors were capable of activating G-proteins, MAPK, as well as DNA synthesis (25). In related studies, the acute stimulation of endothelial nitric oxide synthase (eNOS) by estrogen was reconstituted in COS-7 monkey kidney cells cotransfected with ERα and eNOS, but not by transfection with eNOS alone (23).

Binding molecules for estrogen and progesterone, comprising several molecular species, were isolated from brain synaptosomes by affinity chromatography and characterized by electrophoresis and Western blot (15,81). Microsequencing of one E_2-binding protein indicated that the high-affinity site corresponds to the subunit of an ATPase/ATP synthase. In addition, some studies suggest that estrogen bound to sex hormone–binding globulin, a plasma protein, also binds with specificity to membrane sites recognizing the liganded transport protein (82). These transport-protein interactions promote cAMP generation via the intermediacy of G-proteins. However, further characterization of receptors for such steroid:protein complexes is not available, and it must be recalled that estrogen is in noncovalent association with its plasma protein carrier and dissociates readily therefrom (83).

Binding of progesterone to plasma membrane of amphibian oocytes is specific, saturable, and temperature dependent (31,32). Photoaffinity labeling with the synthetic progestin [^{3}H]-R5020, followed by gel electrophoresis, revealed progestin binding to both 80- and 110-kDa proteins in oocyte cytosol, whereas only the 110-kDa R5020-binding protein was present in oocyte plasma membrane. A progesterone-binding protein (msPR) was identified in crude microsomal, rather than purified plasmalemmal, membranes from porcine liver (84,85). On solubilization, a moderate-affinity site with a dissociation constant (K_d) of 69 nM was found, but, after further purification, affinity decreased to K_d of 228 nM. The final fraction contained two novel peptides of 28 and 56 kDa. Expression of msPR-cDNA in CHO cells led to slightly increased progesterone binding in microsomes, and administration of an antibody against msPR reduced rapid progesterone-initiated Ca^{++} increases in sperm (85). Whether this work represents the first successful cloning

and expression of a steroid receptor associated with cell mem-branes will have to await confirmation. However, Falkenstein et al. *(85)* suggest that the native *plasma* membrane PR may actually be an oligomeric protein complex of about 200 kDa, composed only in part by 28- and 56-kDa peptides.

Glucocorticoids, Aldosterone, and Vitamin D

Progress has been made in the isolation and characterization of plasma membrane receptors for glucocorticoids, aldosterone, and $1,25(OH)_2D_3$, although at this writing, evidence of cloning of the cDNA for any of these proteins is lacking. The membrane glucocorticoid receptor (mGR) was purified from lymphoma cells by immunoaffinity binding with an MAb coupled to Sepharose-4B; the protein displayed properties similar to iGR *(55)*. Scatchard analysis of mGR yielded a K_d of 239 nM and B_{max} of 384 fmol/mg of protein, representing a somewhat higher number of binding sites but a lower affinity than that of the iGR. Peptide maps revealed some sequences that were unique to the membrane form *(55,56)*. Further data suggest that the mGR in lymphoma cells is a transcript variant of the iGR *(56)* (Table 2). Properties of the aldosterone membrane receptor have been analyzed by means of $[^{125}I]$-aldosterone photoaffinity labeling. The protein has an apparent molecular mass of 50 kDa and appears to be distinct from intracellular receptor *(86)*.

The pursuit of membrane receptor for $1,25(OH)_2D_3$ (pmVDR) by affinity isolation has been hampered by the fact that most ligand derivatives lack sufficient binding activity. Nevertheless, work by Baran et al. *(87)* indicates that the vitamin D analog, $[^{14}C]$-1α,25-dihydroxyvitamin D_3 bromoacetate, does exhibit a moderate degree of specific binding to a 36-kDa protein in plasma membranes of rat osteoblast-like cells. Using sequence determination and Western blot, the labeled membrane protein was identified as annexin II, part of a family of membrane-binding proteins previously implicated in the regulation of Ca^{++} signaling, tyrosine phosphorylation, and apoptosis. Partially purified plasma membrane proteins and purified annexin II exhibited specific and saturable binding for $[^3H]$-1α,25(OH)_2 D_3, and antibodies to annexin II inhibited $[^{14}C]$-1α,25(OH)_2 D_3 bromoacetate binding to plasma membranes and also inhibited hormone-induced increases in intracellular calcium in osteoblast-like cells. Hence, these initial results *(87)* suggest that annexin II may serve as a receptor for rapid actions of $1,25(OH)_2D_3$ in rat osteoblast-like cells, but it is not known if this receptor system functions in other cell types. In independent studies, classic biochemical strategies, coupled with analyses of specific binding, were used to isolate the vitamin D membrane receptor (pmVDR) from intestinal epithelium of chicks *(88)*. Basal-lateral membranes were solubilized with detergent and subjected to ion-exchange and gel filtration chromatography. Binding activity eluted with a protein of 65 kDa, with a K_d of 0.7 nM

(88). A highly specific antibody toward plasma membrane VDR failed to recognize the nuclear receptor in Western analyses. On the other hand, a commercially available MAb generated against the "classic" nuclear receptor reacted with many proteins in nuclear fractions of chick intestine, including a band that comigrated with authentic recombinant protein, but did not detect VDR in basolateral membranes *(89)*. Antibody to the plasma membrane receptor, but not to the nuclear receptor, blocked hormonal activation of PKC. The 65-kDa protein was also observed to bind the affinity ligand, $[^{14}C]$-1α,25-dihydroxyvitamin D_3 bromoacetate, and labeling was diminished in the presence of excess nonradioactive ligand *(89)*. Electron microscopic studies of duodena vascularly perfused with control media, $1,25(OH)_2D_3$, or $24,25(OH)_2D_3$ followed by immunochemical staining revealed that $1,25(OH)_2D_3$, but not control media or $24,25(OH)_2D_3$, resulted in dramatically enhanced nuclear localization of the putative membrane receptor *(89)*.

Varied Forms of Steroid Hormone Receptors in Plasma Membranes

Collectively, current findings suggest that membrane receptors for steroid hormones are, in certain instances, transcriptional copies (estrogen) or variants (glucocorticoids) of nuclear receptors and, in other instances, products apparently unrelated to intracellular receptors (aldosterone and vitamin D). There is evidence for alternatively spliced transcripts of several steroid receptors, and these variant receptors give rise to proteins of different molecular size and, possibly, modified properties *(56)*. Membrane insertion of receptors in primary transcript form would likely require one or more hydrophobic regions, and post-translational modification of receptor protein leading to cell membrane targeting may also occur, including phosphorylation, glycosylation, and addition of lipid anchors or other modifications, such as palmitoylation or myristoylation. Surface steroid hormone receptors may also be part of a mul timeric complex including a "classic" nuclear receptor but bound to as-yet-unidentified transmembrane proteins and coupled to membrane-associated signaling molecules *(3,7, 15,79)*. Alternatively, plasma membrane receptors for steroids may have several common structural features with, but may be distinct from, the intracellular steroid hormone receptors *(88,89)*. In the case of retinoic acid and estradiol, binding to known membrane proteins, such as M6P/IGF-2 receptor *(75)* or HER-2 receptor *(90)*, respectively, may modulate some ligand effects. Progesterone appears to interact directly with oxytocin receptor, a G-linked protein at the cell surface, and inhibits some functional effects of oxytocin signaling, thus suppressing uterotonic activity of oxytocin *(91)*. Progesterone congeners also bind with moderate affinity to γ-aminobutyrate type A ($GABA_A$) receptors that comprise ligand-gated ion channel complexes *(4, 28)*. Absence of the γ-subunit of $GABA_A$ receptor in appropriate knockout mice results in a significant decrease in

sensitivity to neuroactive steroids such as pregnanolone *(92)*. Similarly, acute vascular relaxation induced by pharmacologic levels of E_2 may be mediated by its binding to the regulatory subunit of Maxi-K channels in membranes *(93)*, thus supporting the view that some effects of steroids, at least at high micromolar concentration, may be mediated by known membrane receptors with previously unrecognized steroid-binding sites.

Using ERα gene knockout (ERKO) mice, Gu et al. *(94)* showed that rapid actions of estradiol at 50 nM on kainate-induced currents in hippocampal neurons still occur, and the effect is not inhibited by ICI 182,780, a pure antagonist of hormone binding to both ERα and ERβ. These investigators suggest that a distinct estrogen-binding site exists in neurons and appears to be coupled to kainate receptors by a cAMP-dependent process. However, it is important to note that alternatively spliced forms of ERα *(95)*, as well as ERβ *(96)*, can occur in ERKO mice, thus complicating the interpretation of these results. Moreover, uterine tissues of ovariectomized ERKO mice exhibit 5–10% of the estradiol binding present in wild-type uteri *(95,97)*, and the significance of these residual estrogen-binding sites in ERKO target cells is unclear. Nonetheless, further development of double ERα and ERβ gene knockouts and perfection of this new technology should prove important in deciphering the contribution of "classic" and novel receptor forms in hormone action.

In future work, it will be important to pursue isolation and characterization of constituent proteins from homogeneous plasma membranes prepared in the presence of proteinase inhibitors *(18,76,98)*. Verification of their purity should be confirmed by use of a balance sheet for enzyme or other membrane markers *(18,76)*. Screening for activity of receptor would benefit from the use of independent approaches, such as ligand binding with radio- or photoaffinity-labeled steroids and immunoassay directed toward known intracellular receptors *(15,31,55,78,86)*. These several approaches may detect membrane receptors originating from a transcript other than that of intracellular receptor. As with the mixed steroid hormone–binding protein systems known to occur within cells and in their extracellular fluids, it may well be that multiple forms of receptor proteins for steroids coexist in plasma membranes, thus complicating efforts to isolate and characterize the individual binding species in this cell compartment. Our efforts to understand ligand-receptor interactions are often limited by simplistic "lock-and-key" models that may not accurately reflect the true state of complex molecular signaling cascades. Study of the molecular organization of several neurotransmitter receptor families has already shown that extraordinary biologic variability occurs, with multiple "keys" and multiple "locks" sometimes involved in ligand-receptor recognition *(99)*. We must consider the existence of similar high-affinity, but possibly multivalent and multifunctional, receptors in the steroid hormone superfamily *(75,91–93)*.

Perspectives

Ever since the discovery of chromosomal puff induction by ecdysone, cell regulation by steroid hormones has focused primarily on a nuclear mechanism of action. However, even the venerable steroid hormone ecdysone elicits rapid membrane effects that may facilitate later nuclear alterations *(100)*. Indeed, membrane-initiated responses appear to be the cell's earliest response to steroids and may be prerequisite to subsequent genomic responses *(2,3,7, 10;* see also Fig. 1). Coupling of surface membrane, cytoplasmic, and nuclear responses may offer a progressive, ordered expansion of initial signal. Accordingly, the terms *genomic* and *nongenomic* may not accurately define such a response continuum *(101)*. Future investigations should focus on potential interactions of membrane and nuclear steroid receptors that may promote activation of transcription and other specific hormonal responses. Molecular details of cross-communication between steroid and peptide receptors are also beginning to emerge *(3,98)*, and membrane steroid receptors may be in a pivotal location to promote convergence among diverse signaling pathways (Fig. 1). Indeed, the consequences of steroid hormone recognition at the outer cell membrane of target, but not nontarget, cells are shared by numerous other classes of regulatory molecules (cf. ref. *102*), including peptide hormones, neurotransmitters, drugs, plant lectins, mitogens, and antibodies *(3)*. Although the agonists are manifold, the signaling mechanisms are few. Primary signal recognition at the surface would be fleeting, but the mutual specificities and affinities are high, and thus sufficient for setting the appropriate signal transduction chain in motion. However, until the current surge of renewed focus on this problem, identification of these instantaneous triggering interactions for steroid hormones has accumulated relatively slowly, having been limited by technical and microanalytic barriers that are now being surmounted.

Ligand-receptor interactions depend on an extensive array of extracellular and intracellular partners to localize to membrane microdomains, recruit signaling molecules, and trigger intracellular signaling pathways. As the consequences of surface interactions are analyzed in greater depth, it will be important to evaluate further the biologic role of rapid internalization of steroid-binding sites from plasma membranes via endocytotic-lysosomal pathways *(2,3,88,101,103–105)*. These membrane-initiated events may involve cytostructural elements or scaffold proteins that contribute to signal propagation to the nucleus and the nuclear-protein matrix *(2,101,104–107;* Fig. 1). Thus, antibodies specific to intestinal membrane VDR reveal a vitamin D–induced redistribution of membrane receptor, a protein that appears distinct from intracellular receptor, to the nucleus within 5 min of binding ligand *(89)*. It is unknown whether the membrane receptor has inherent DNA- or coregulator-binding capacity to alter transcription; alter-
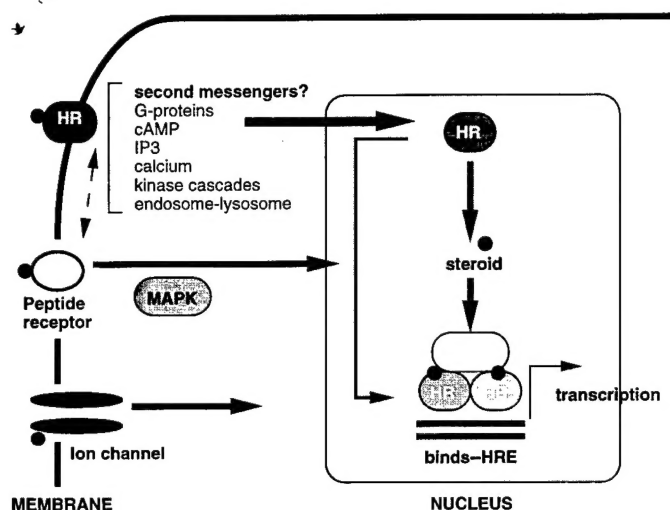
Fig. 1. Postulated mechanism of action of steroid hormones (black circles) in target cells with steroid hormone receptor (HR). In most current models, steroid binding to HR is believed to promote alterations in receptor conformation favoring enhanced association with coactivator proteins and with specific hormone-responsive elements (HRE) in the nucleus, leading, in turn, to initiation of selective gene transcription. However, the latter model fails to account for numerous, rapid cell responses to steroid treatment (see Table 1 and text). These deficiencies in the genomic model of hormone action require integration with the latter observations. In the model shown here, steroids may also bind to a membrane HR, with potential for promotion of hormonal responses via a complementary pathway that may cross-communicate or interact directly with the genomic mechanism. As noted in the text, membrane HR may be known molecules (kinases, ion channels, other receptors) with previously unrecognized binding sites for steroid, new isoforms of HR in membranes, "classic" forms of HR complexed with other membrane-associated proteins, truly novel membrane proteins, or a combination of these. Available evidence indicates that liganded membrane HR may affect one or more of several pathways, including modulation of ion channels, leading to enhanced flux of ions, notably Ca^{++}; interaction with peptide membrane receptors; and activation of G-proteins, nucleotide cyclases, and MAPK, with resultant increases in their catalytic products (see Table 1). These membrane interactions may promote phosphorylation of HR itself via steroid-induced or ligand-independent pathways. The intricate array of physiologic responses of cells to steroid hormones may occur as a consequence of a synergistic feed-forward circuit in which steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of HR (Table 1). Active reconsideration of the unqualified genomic model of nuclear receptor action is ongoing, and the probable importance of alternate signaling pathways elicited by surface recognition is now increasingly evident.

natively, it could serve to shuttle ligand to the nuclear-localized fraction of receptor. As has frequently been noted from these laboratories (cf. ref. *105*), the cellular mechanisms governing the further transport and targeting of signaling molecules are powerful avenues of current investigation.

Many issues remain to be resolved for fuller understanding of the biologic actions of steroid hormones. Foremost among these is the structural characterization of membrane steroid hormone receptors. It is now abundantly clear that the nuclear receptor–mediated mechanism as the sole means by which steroid hormones act is incomplete *(2,3,5,7,15, 107)*. It is likewise unmistakable that membrane effects of steroid hormones represent an established phenomenon that is by no means to be construed as alternative to the genomic pathway, and that demands continued investigation. Indeed, the chain of membrane-initiated events is helping to account for the relatively prolonged, apparent silence between the capture of the hormone at the surface of its preferential target and the eventual outcome in augmented genomic activities. In challenging the dogma that steroid hormones act exclusively via intracellular receptors, the membrane receptor experiments reviewed here provide a persuasive paradigm for a potentially new class of drugs for human therapy. The clinical use of steroid hormone agonists and antagonists has substantially changed the course of many hormone-related diseases, but side effects of many agents currently in use are also significant. In-depth analysis of the relative contributions of nuclear and membrane-initiated activities in steroid receptor biology may lead to the development of pharmaceutical agents that exert differential activities in the two pathways, thus favoring more selective drug delivery and promoting the emergence of novel approaches for treatment of many cell metabolic and proliferative defects.

Acknowledgments

References

1. Evans, R. M. (1988). *Science* **240**, 889–895.
2. Szego, C. M. (1984). *Life Sci.* **35**, 2383–2396.
3. Szego, C. M. and Pietras, R. J.. (1981). In: *Biochemical actions of hormones.* vol. 8. Litwack, G. (ed.). Academic: New York.
4. Moss, R. L., Gu, Q., and Wong, M. (1997). *Recent Prog. Horm. Res.* **52**, 33–70.
5. Nemere, I. and Farach-Carson, M. (1998). *Biochem. Biophys. Res. Commun.* **248**, 443–449.
6. Christ, M., Haseroth, K., Falkenstein, E., and Wehling, M. (1999). *Vitam Horm.* **57**, 325–373.
7. Watson, C. S. and Gametchu, B. (1999). *Proc. Soc. Exp. Biol. Med.* **220**, 9–19.
8. Milgrom, E., Atger, M., and Baulieu, E. E. (1973). *Biochim. Biophys. Acta* **320**, 267–283.
9. Pietras, R. J. and Szego, C. M. (1975). *Nature* **253**, 357–359.
10. Improta-Brears, T., Whorton, A. R., Codazzi, F., York, J. D., Meyer, T., and McDonnell, D. P. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 4686–4691.

11. Szego, C. M. and Davis, J. S. (1967). *Proc. Natl. Acad. Sci. USA* **58**, 1711–1718.
12. Aronica, S., Kraus, W., and Katzenellenbogen, B. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 8517–8521.
13. Endoh, H., Sasaki, H., Maruyama, K., Takeyama, K., Waga, I., Shimizu, T., Kato, S., and Kawashima, H. (1997). *Biochem. Biophys. Res. Commun.* **235**, 99–102.
14. Le Mellay, V., Grosse, B., and Lieberherr, M. (1997). *J. Biol. Chem.* **272**, 11,902–11,907.
15. Ramirez, V. D. and Zheng, J. (1996). *Front. Neuroendocrinol.* **17**, 402–439.
16. Pietras, R. J. and Szego, C. M. (1977). *Nature* **265**, 69–72.
17. Pietras, R. J. and Szego, C. M. (1979). *J. Cell. Physiol.* **98**, 145–159.
18. Pietras, R. J. and Szego, C. M. (1980). *Biochem. J.* **191**, 743–760.
19. Selye, H. (1942). *Endocrinology* **30**, 437–453.
20. Dufy, B., Partouche, C., Poulain, D., Dufy-Barbe, L., and Vincent, J. (1976). *Neuroendocrinology* **22**, 38–47.
21. Kelly, M. J., Moss, R. L., and Dudley, C. A. (1977). *Exp. Brain Res.* **30**, 53–64.
22. Mendelsohn, M. and Karas, R. (1999). *N. Engl. J. Med.* **340**, 1801–1811.
23. Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M., and Shaul, P. (1999). *J. Clin. Invest.* **103**, 401–406.
24. Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K., and Jo, I. (1999). *Biochem. Biophys. Res. Commun.* **263**, 257–262.
25. Razandi, M., Pedram, A., Greene, G., and Levin, E. (1999). *Mol. Endocrinol.* **13**, 307–319.
26. Berthois, Y., Pourreau-Schneider, N., Gandilhon, P., Mittre, H., Tubiana, N., and Martin, P. M. (1986). *J. Steroid Biochem.* **25**, 963–972.
27. Stevis, P., Deecher, D., Suhadolnik, L., Mallis, L., and Frail, D. (1999). *Endocrinology* **140**, 5455–5458.
28. McEwen, B. S. (1991). *Trends Pharmacol. Sci.* **12**, 141–147.
29. Godeau, J., Schorderet-Slatkine, S., Hubert, P., and Baulieu, E. E. (1978). *Proc. Natl. Acad. Sci. USA* **75**, 2353–2357.
30. Kostellow, A. B., Weinstein, S. P., and Morrill, G. A. (1982). *Biochim. Biophys. Acta* **720**, 356–363.
31. Sadler, S. and Maller, J. (1982). *J. Biol. Chem.* **257**, 355–361.
32. Morrill, G. A. and Kostellow, A. B. (1999). *Steroids* **64**, 157–167.
33. Blakemore, P., Neulen, J., Lattanzio, F., and Beebe, S. (1991). *J. Biol. Chem.* **266**, 18,655–18,659.
34. Meizel, S. and Turner, K. (1991). *Mol. Cell. Endocrinol.* **77**, 1–5.
35. Sabeur, K., Edwards, D., and Meizel, S. (1996). *Biol. Reprod.* **54**, 993–1001.
36. Gorczynska, E. and Handelsman, D. (1995). *Endocrinology* **136**, 2052–2059.
37. Peterziel, H., Mink, S., Schonert, A., Becker, M., Klocker, H., and Cato, A. C. (1999). *Oncogene* **18**, 6322–6329.
38. Perusquia, M. and Villalon, C. (1999). *Eur. J. Pharmacol.* **371**, 169–178.
39. Lieberherr, M. and Grosse, B. (1994). *J. Biol. Chem.* **269**, 7217–7223.
40. Benten, W., Lieberherr, M., Stamm, O., Wrehlke, C., Guo, Z., and Wunderlich, F. (1999). *Mol. Biol. Cell* **10**, 3113–3123.
41. Hummerick, H. and Soboll, S. (1989). *Biochem. J.* **258**, 363–367.
42. Segal, J. (1989). *Endocrinology* **124**, 2755–2764.
43. Segal, J., Masalha, S., Schwalb, H., Merin, G., Borman, J. B., and Uretzky, G. (1996). *J. Endocrinol.* **149**, 73–80.
44. Davis, P. and Davis, F. (1996). *Thyroid* **6**, 497–504.
45. Lakatos, P. and Stern, P. (1991). *Acta Endocrinol. (Copenh.)* **125**, 603–608.
46. Roussel, J. P., Grazzini, E., Zumbihl, R., Rodriguez, E., and Astier, H. (1995). *Eur. J. Pharmacol.* **289**, 205–215.
47. Alderson, R., Pastan, I., and Cheng, S.-Y. (1985). *Endocrinology* **116**, 2621–2630.
48. Pontecorvi, A., Lakshmanan, M., and Robbins, J. (1987). *Endocrinology* **121**, 2145–2152.
49. Estupina, C., Belmar, J., Tapia-Arancibia, L., Astier, H., and Arancibia, S. (1997). *Exp. Brain Res.* **113**, 337–342.
50. Towle, A. C. and Sze, P. Y. (1983). *J. Steroid Biochem.* **1**, 135–143.
51. Orchinik, M., Murray, T., and Moore, F. (1991). *Science* **252**, 1848–1851.
52. Andres, M., Marino, A., Macarulla, J., and Trueba, M. (1997). *Cell. Mol. Life Sci.* **53**, 673–680.
53. Gametchu, B. (1987). *Science* **236**, 456–461.
54. Gametchu, B., Watson, C. S., and Wu, S. (1993). *FASEB J.* **7**, 1283–1292.
55. Powell, C., Watson, C., and Gametchu, B. (1999). *Endocrine* **10**, 271–280.
56. Chen, F., Watson, C., and Gametchu, B. (1999). *J. Cell. Biochem.* **74**, 418–429.
57. Ebata, S., Muto, S., Okada, K., Nemoto, J., Amemiya, M., Saito, T., and Asano, Y. (1999). *Kidney Int.* **56**, 1400–1412.
58. Doolan, C. M. and Harvey, B. J. (1996). *J. Biol. Chem.* **271**, 8763–8767.
59. Kolbel, F. and Schreiber, V. (1996). *Mol. Cell. Biochem.* **160/161**, 111–115.
60. Doris, P., Hayward-Lester, A., Bourne, D., and Stocco, D. (1996). *Endocrinology* **137**, 533–539.
61. LaBella, F. and Templeton, J. (1998). *Clin. Exp. Hypertens.* **20**, 601–609.
62. Huang, L., Li, H., and Xie, Z. (1997). *J. Mol. Cell. Cardiol.* **29**, 429–437.
63. Caffrey, J.M. and Farach-Carson, M.C. (1989). *J. Biol. Chem.* **264**, 20,265–20,274.
64. Boyan, B. D., Sylvia, V. L., Dean, D. D., Pedrozo, H., Del Toro, F., Nemere, I., Posner, G. H., and Schwartz Z. (1999). *Steroids* **64**, 129–136.
65. Nemere, I., Schwartz, Z., Pedrozo, H., Sylvia, V. L., Dean, D. D., and Boyan, B. D. (1998). *J. Bone Miner. Res.* **13**, 1353–1359.
66. Jespersen, B., Randlov, A., Abrahamsen, J., Fogh-Andersen, N., Olsen, N. V., and Kanstrup, I. L. (1998). *Am. J. Hypertens.* **11**, 659–666.
67. Berry, D. M. and Meckling-Gill, K. A. (1999). *Endocrinology* **140**, 4779–4788.
68. Kajikawa, M., Ishida, H., Fujimoto, S., Mukai, E., Nishimura, M., Fujita, J., Tsuura, Y., Okamoto, Y., Norman, A. W., and Seino, Y. (1999). *Endocrinology* **140**, 4706–4712.
69. Nemere, I. (1999). *J. Bone Miner. Res.* **14**, 1543–1549.
70. Bianchi, M. L., Ardissino, G. L., Schmitt, C. P., Dacco, V., Barletta, L., Claris-Appiani, A., and Mehls, O. (1999). *J. Bone Miner. Res.* **14**, 1789–1795.
71. O'Connell, M., Chua, R., Hoyos, B., Buck, J., Chen, Y., Derguini, F., and Hammerling, U. (1996). *J. Exp. Med.* **184**, 549–555.
72. Sundaram, M., Sivaprasadarao, A., DeSousa, M. M., and Findlay, J. B. (1998). *J. Biol. Chem.* **273**, 3336–3342.
73. Christensen, E. I., Moskaug, J. O., Vorum, H., Jacobsen, C., Gundersen, T. E., Nykjaer, A., Blomhoff, R., Willnow, T. E., and Moestrup, S. K. (1999). *J. Am. Soc. Nephrol.* **10**, 685–695.
74. Nykjaer, A., Dragun, D., Walther, D., Vorum, H., Jacobsen, C., Herz, J., Melsen, F., Christensen, E. I., and Willnow, T. E. (1999). *Cell* **96**, 507–515.
75. Kang, J., Li, Y., and Leaf, A. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 13,671–13,676.
76. Pietras, R. J. and Szego, C. M. (1979). *J. Steroid Biochem.* **11**, 1471–1483.
77. Monje, P. and Boland, R. (1999). *Mol. Cell. Endocrinol.* **147**, 75–84.

78. Pappas, T., Gametchu, B., and Watson, C. (1995). *FASEB J.* **9**, 404–410.

79. Russell, K. S., Haynes, M., Sinha, D., Clerisme, E., and Bender, J. R. (2000). *Proc. Natl. Acad. Sci. USA* **97**, 5930–5935.

80. Norfleet, A., Thomas, M., Gametchu, B., and Watson C. (1999). *Endocrinology* **140**, 3805–3814.

81. Zheng, J. and Ramirez, V. (1997). *J. Steroid Biochem. Mol. Biol.* **62**, 327–336.

82. Rosner, W., Hryb, D. J., Khan, M., Nakhla, A. M., and Romas, N. A. (1999). *Steroids* **64**, 100–106.

83. Szego, C. and Roberts, S. (1946). *Proc. Soc. Exp. Biol. Med.* **61**, 161–164.

84. Meyer, C., Schmid, R., Scriba, P., and Wehling, M. (1996). *Eur. J. Biochem.* **239**, 726–731.

85. Falkenstein E., Heck, M., Gerdes, D., Grube, D., Christ, M., Weigel, M., Buddhikot, M., Meizel, S., and Wehling, M. (1999). *Endocrinology* **140**, 5999–6002.

86. Eisen, C., Meyer, C., Christ, M., Theisen, K., and Wehling, M. (1994). *Cell. Mol. Biol.* **40**, 351–358.

87. Baran, D. T., Quail, J. M., Ray, R., Leszyk, J., and Honeyman, T. (2000). *J. Cell. Biochem.* **78**, 34–46.

88. Nemere, I., Dormanen, M., Hammond, M., Okamura, W., and Norman, A. (1994). *J. Biol. Chem.* **261**, 16,106–16,114.

89. Nemere, I., Ray, R., and McManus, W. (2000). *Am. J. Physiol. Endocr. Metab.* **278**, E1104–E1114.

90. Matsuda, S., Kadowaki, Y., Ichino, M., Akiyama, T., Toyoshima, K., and Yamamoto, T. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 10,803–10,808.

91. Grazzini, E., Guillon, G., Mouillac, B., and Zingg, H. H. (1998). *Nature* **392**, 509–512.

92. Mihalek, R. M., Banerjee, P. K., Korpi, E. R., et al. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 12,905–12,910.

93. Valverde, M., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M. I., Mann, G. E., Vergara, C., and Latorre, R. (1999). *Science* **285**, 1929–1931.

94. Gu, Q., Korach, K., and Moss, R. (1999). *Endocrinology* **140**, 660–666.

95. Couse, J., Curtis, S. W., Washburn, T. F., Lindzey, J., Golding, T. S., Lubahn, D. B., Smithies, O., and Korach, K. S. (1995). *Mol. Endocrinol.* **9**, 1441–1454.

96. Kuiper, G. G., Enmark, E., Pelto-Huikko, E., Nilsson, S., and Gustafsson, J. A. (1996). *Proc. Natl. Acad. Sci. USA* **93**, 5925–5930.

97. Lubahn, D. B., Mouger, J., Golding, T., Couse, J., Korach, K., and Smithies, O. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 11,162–11,166.

98. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. (1995). *Oncogene* **10**, 2435–2446.

99. Civelli, O. (1995). *J. Recept. Signal Transduct. Res.* **15**, 161–172.

100. Schneider, S., Wunsch, S., Schwab, A., and Oberleithner, H. (1996). *Mol. Cell. Endocrinol.* **116**, 73–79.

101. Szego, C. M. (1994). *Endocrine* **2**, 1079–1093.

102. Ehrlich, P. (1900). In: *The collected papers of Paul Ehrlich.* vol. II (1957), Himmelweit, F. (ed.). Pergamon: Oxford.

103. Williams, M. and Baba, W. (1967). *J. Endocrinol.* **39**, 543–554.

104. Pietras, R. J. and Szego, C. M. (1984). *Biochem. Biophys. Res. Commun.* **123**, 84–90.

105. Szego, C. M. and Pietras, R. J. (1984). *Int. Rev. Cytol.* **88**, 1–302.

106. Szego, C. M., Sjöstrand, B. M., Seeler, B. J., Baumer, J., and Sjöstrand, F. S. (1988). *Am. J. Physiol.* **254** (**Endocrinol. Metab.** *17*), E775–E785.

107. Chen, Y.-Z. and Qui, J. (1999). *Mol. Cell Biol. Res. Commun.* **2**, 145–149.

108. Suyemitsu, T. and Terayama, H. (1975). *Endocrinology* **96**, 1499–1508.

109. Nemere, I. and Szego, C. M. (1981). *Endocrinology* **108**, 1450–1462.

110. Pourreau-Schneider, N., Berthois, Y., Gandilhon, P., Cau, P., and Martin, P. M. (1986). *Mol. Cell. Endocrinol.* **48**, 77–88.

111. Wehling, M., Kasmayr, J., and Theisen, K. (1991). *Am. J. Physiol.* **260**, E719–E726.

112. Rambo, C. O. and Szego, C. M. (1983). *J. Cell Biol.* **97**, 679–685.

113. Simoncini, T., Hafezi-Moghadam, A., Brazil, D., Ley, K., Chin, W., and Liao, J. (2000). *Nature* **407**, 538–541.

114. Watters, J. J., Chun, T.-Y., Kim, Y.-N., Bertics, P. J., and Gorski, J. (2000). *Mol. Endocrinol.* **14**, 1872–1881.